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TITLE: Identification of the Microtubule Inhibitor-Activated Bcl-xL Kinase: A Regulator of Breast Cancer Cell Chemosensitivity to Taxol

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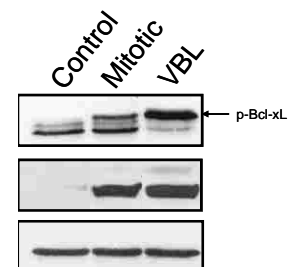
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14. ABSTRACT This training grant set out to define a molecular pathway involved in breast tumor resistance to the key chemotherapeutic class of drugs called microtubule inhibitors (MTIs). It is also designed to train the Principal Investigator (PI) as a future breast cancer physician-scientist. MTIs are the most actively used agents for metastatic and adjuvant BC therapy, yet their use is limited by resistance. They activate a kinase that phosphorylates and inactivates Bcl-xL, an anti-apoptotic protein that causes resistance to chemotherapeutic agents. The goal is to identify the Bcl-xL kinase first by developing an <i>in vitro</i> assay for it. Data presented here shows that a 12 amino acid peptide, termed FL62, that harbors the Bcl-xL phosphorylation site Ser62, is an excellent substrate for the MTI activated kinase. Activation of this kinase correlates with Bcl-xL phosphorylation as well as mitotic arrest and cyclin B expression, implicating cyclin-dependent kinase-1 (Cdk1). When tested, purified Cdk1 and cellular Cdk1/cyclin B immunopurified from mitotic and vinblastine treated cells robustly phosphorylated FL62 confirming that Cdk1/cyclin B is the FL62 kinase. This provides the foundation to validate Cdk1/cyclin B as the MTI activate Bcl-xL kinase <i>in vivo</i> .					
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**INTRODUCTION:** This training grant set out to define a molecular pathway involved in breast tumor resistance to the key chemotherapeutic class of drugs called microtubule inhibitors (MTIs). It is also designed to train the Principal Investigator (PI) as a future breast cancer physician-scientist. MTIs are the most actively used agents for metastatic and adjuvant BC therapy, yet their use is limited by resistance (1-4). Bcl-xL is an anti-apoptotic protein that causes resistance to chemotherapeutic agents when overexpressed in many tumor types. Bcl-xL is phosphorylated and inactivated following MTI treatment (5) and may represent a molecular pathway that leads to resistance when inactivated. The goal to identify the Bcl-xL kinase by developing an *in vitro* assay for it and, using that assay, purify and identify the enzyme. The ultimate goal is to investigate any correlation between Bcl-xL kinase activity, Bcl-xL phosphorylation, and tumor cell sensitivity to MTIs in human breast tumor cells. Concurrently, a specific breast cancer training plan will facilitate the PI's professional development as a future BC physician-scientist. Training includes clinical research and medical student training, a monthly Breast Cancer Focus Group formed by the PI, research seminars, dissertation writing, publication, and presentations at professional meetings and departmental seminars.

**BODY:** In **Specific Aim 1**, a robust and specific assay for Bcl-xL kinase activity was developed and optimized using a 12 amino acid peptide FL62 harboring the MTI-induced phosphorylation site Ser62 in Bcl-xL. The following experiments prove that we are assaying the kinase responsible for MTI-induced cellular Bcl-xL phosphorylation, and that its properties corresponded to Cdk1/cyclin B. Only two select figures are shown, while other experiments are described due to space constraints.

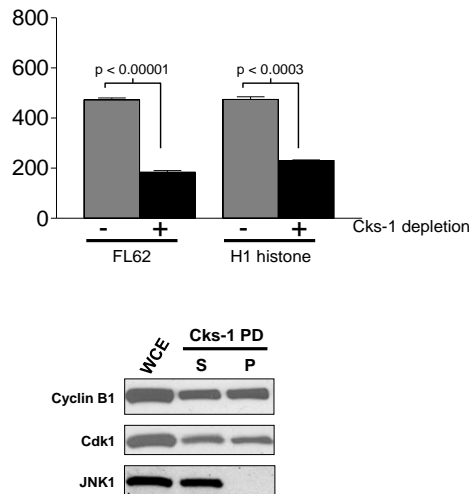


**Figure 1.** Partial Bcl-xL phosphorylation and FL62 kinase activation during normal mitosis. Extracts were prepared from untreated asynchronous KB-3 cells (Control), KB-3 cells synchronized in mitosis (mitotic) by double thymidine block, or asynchronous KB-3 cells treated with 30 nM vinblastine for 16 h. FL62 kinase activity was assayed and plotted as specific activity (mean  $\pm$  s.d.,  $n = 3$ ). The extracts were also immunoblotted for Bcl-xL and cyclin B.

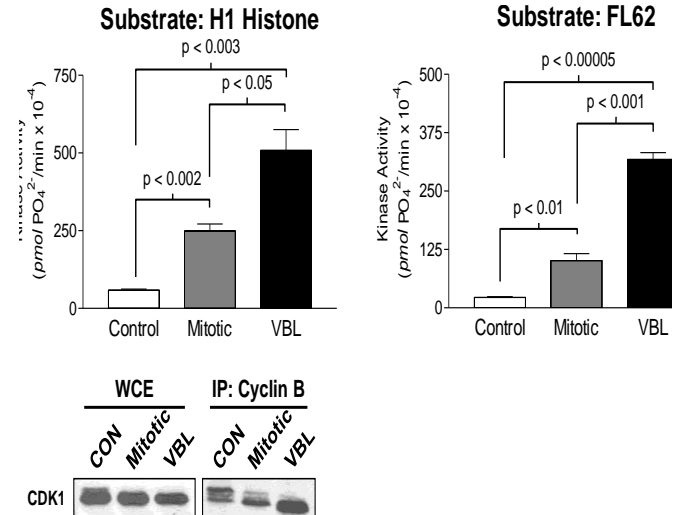
First, FL62 kinase

was activated by all MTIs tested including vinblastine, paclitaxel, vincristine, and colchicine (data not shown), known inducers of Bcl-xL phosphorylation (3). However, the DNA damaging agent doxorubicin, which does not induce Bcl-xL phosphorylation (3), did not activate FL62 kinase (data not shown). To validate the assay biologically, FL62 kinase activity was tested over a range of vinblastine concentrations. FL62 kinase activity paralleled the extent of cellular Bcl-xL phosphorylation, reaching maximal levels from 10-300 nM vinblastine, and, interestingly, tightly paralleled the expression level of cyclin B (data not shown). Investigation of cells synchronized in mitosis showed a significant 4-fold increase in FL62 kinase activity and those treated vinblastine showed a 13-fold increased activity (Fig. 1). Bcl-xL phosphorylation also followed the same pattern (Fig. 1), and to our knowledge, this is the first data showing phosphorylation of Bcl-xL during normal mitosis.

Three experiments demonstrate that FL62 is Cdk1/cyclin B. First, commercially available purified Cdk1 (data not shown) phosphorylated FL62 as well as full length Bcl-xL uniquely and specifically on Ser62 (data not shown). Second, Cdk1/cyclin B immunopurified from mitotic and vinblastine treated KB-3 cells phosphorylated



**Figure 2.** Total Cdk activity was depleted from extracts of vinblastine treated KB-3 cells using Cdc binding subunit protein conjugated to agarose. FL62 and the Cdk positive control substrate H1 histone were then phosphorylated with original or Cdk depleted extract. Immunoblots of the original extract and supernatant (S) and pellet (P) are shown. JNK was immunoblotted as a negative control (mean  $\pm$  s.d., n = 3).



**Figure 3.** Cdk1/cyclin B was immunopurified from extract from control, mitotic, and vinblastine treated cells using cycl. B antibody. Immunoprecipitated Cdk1/cyclin B was then used to phosphorylate FL62 or H1 histone and kinase activity plotted (mean  $\pm$  s.d., n = 3)

FL62 (Fig. 2) with the same pattern observed in Fig. 1. Lastly, depletion of Cdk1 from mitotic and vinblastine treated KB-3 cells quantitatively depleted FL62 kinase activity (Fig. 3).

We are confident that the Bcl-xL kinase is Cdk1/cyclinB, which partially covers **Specific Aim 2**, identification of the Bcl-xL kinase *in vivo*. Experiments are underway using Cdk inhibitors to validate that it is indeed the *in vivo* Bcl-xL kinase.

**Specific training tasks:** My training proceeded as planned in the original proposal. Scientifically, I presented two research seminars to the Department of Biochemistry & Molecular Biology (2008-2009); two research presentations to my thesis Permanent Advisory Committee; attended a student seminar course (Fall 2008); presented a poster at the UAMS Student Research Day (spring 2009); co-authored a manuscript accepted by the *Journal of Biological Chemistry* (2008) using data from the current project; and continue to work on identifying Cdk1/cyclin B as the *in vivo* Bcl-xL kinase. A preliminary manuscript is in preparation and submission is pending more results. I successfully defended my dissertation orally and have submitted a written thesis that is pending approval. For career development, I presented a poster and gave an oral presentation at two professional meetings. Clinically, I co-authored a clinical protocol approved by the UAMS Institutional Review Board (Protocol #109857), began medical school rotations; recently completed a one month surgical oncology rotation (Fall 2009); and continue to organize monthly meetings for UAMS's Breast Cancer Focus Group.

## KEY RESEARCH ACCOMPLISHMENTS:

### **Basic Research**

- Development of a robust and specific Bcl-xL kinase assay using the peptide substrate FL62
- FL62 kinase activity correlates with mitotic arrest, Bcl-xL phosphorylation, and cyclin B expression
- FL62 kinase is the Cdk1/cyclin B1 complex
  - Purified Cdk1 phosphorylates FL62 and full-length Bcl-xL on Ser62 *in vitro*
  - Activated Cdk1/cyclin B immunopurified from mitotic and vinblastine treated cells phosphorylates FL62
  - Depletion of Cdk from extracts of mitotic and vinblastine treated cells quantitatively depletes FL62 kinase activity

### **Clinical Research**

- Co-author of the clinical research protocol “Alterations in the Bcl-2 Family Proteins in the Peripheral Blood Following Treatment with Taxanes in Patients with Breast Cancer.” Accepted by the UAMS Institutional Review Board (**Study protocol #109857**)

## REPORTABLE OUTCOMES:

### **Publications, Posters, and Presentations**

- Upreti M, Galitovskaya EN, Chu R, Tackett AJ, **Terrano DT**, Granell S, Chambers TC. Identification of the major phosphorylation site in Bcl-xL induced by microtubule inhibitors and analysis of its functional significance. *J. Biol. Chem.*, 2008, 283(51), 35517-25.
- **David Terrano\*** and Timothy C. Chambers. CDK1-mediated phosphorylation and inactivation of Bcl-xL/Bcl-2: A functional link between mitotic arrest and apoptosis. Abstract 3865. \*Oral presentation at the Minisymposium “Mechanisms of Drugs Targeting Cell Cycle Controls”. AACR 100th Annual Meeting 2009, Denver, CO.
- **Terrano, DT**, Upreti, M, and Chambers TC. A functional link between mitotic arrest and apoptosis: CDK1-mediated phosphorylation and inactivation of Bcl-xL/Bcl-2, Abcam Mitosis and Cancer Symposium, Oral presentation given by Dr. Chambers, Amsterdam, Netherlands, February 27, 2009,
- **David Terrano\***, Jessica J. Joheim, Laura F. Hutchins, Issam Makhoul, and Timothy C. Chambers. CDK1 mediated Bcl-xL phosphorylation links mitotic arrest to apoptosis. Abstract Number 112. The American Society of Clinical Investigation/American Association of Physician Scientists Joint Meeting, 2009, Chicago, IL. \*Selected for Travel Award by ASCI.
- **Terrano, DT**. “Cdk1 phosphorylation of anti-apoptotic Bcl-xL as a new link between mitotic arrest and apoptosis,” 2008, Department of Biochemistry & Molecular Biology seminar (twice).

### **Clinical Activities**

- As the lead organizer of the UAMS Breast Cancer Focus Group (BCFG), I invite a basic or clinical researcher each month to present their breast cancer research

### **Clinical Activities**

- Surgical clinical rotation as part of the MD training of his MD PhD.
- Co-author the clinical protocol #109857 “Alterations in the Bcl-2 Family Proteins in the Peripheral Blood Following Treatment with Taxanes in Patients with Breast Cancer.”

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